(19) [Publishing Country] JAPAN PATENT OFFICE(JP)

(12) [Kind of Publication] laid-open disclosure public patent bulletin(A)

(11) [Publication Number] JP 11-152228

(43) [Publication Date] June 8, 1999

(54) [Title of the Invention] TSC-22 Inducer

(51) [IPC, 6th edition]

A61K 31/495 AED
ABJ
ABN
ACV
ADT

// C07D215/22

[FI]

A61K 31/495 AED
ABJ
ABN
ACV
ADT

C07D215/22

[Examination Request] not requested

[Number of Claims] 2

[Application Form] FD

[Total number of pages] 17

(21) [Filing Number] JP 9-336516

(22) [Filing Date] November 19, 1997

(71) [Applicant]

[Identification Number] 000206956

[Name] OTSUKA PHARMACEUT CO LTD

[Address] 9-banchi, Kandatsukasamachi 2-chome, Chiyoda-ku, Tokyo

(72) [Inventor]

[Name] KAWAMATA HITOSHI

[Address] 703, LIONS MANSION Tokushima-minamisako, 6-12, Minamisako 6-bancho, Tokushima-shi, Tokushima

(72) [Inventor]

[Name] SATO MITSUNOBU

[Address] 187-10, Shimofukuman, Hachimancho, Tokushima-shi, Tokushima

(74) [Attorney]

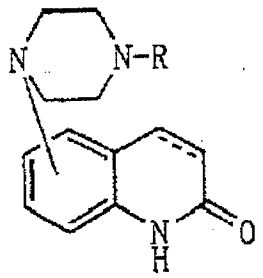
[Patent Attorney]

[Name] SAEGUSA EIJI (ten others)

(54) [Title of the Invention] TSC-22 Inducer

(57) [Abstract]

[Constituent] The present invention provides a TSC-22 inducer whose active ingredient is a carbostyril derivative represented by the general formula [FORMULA 1]:

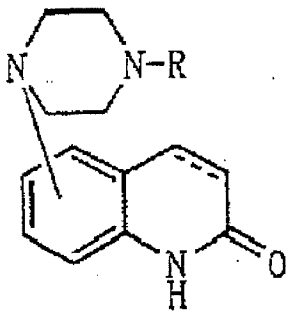


[wherein R represents a benzoyl group that may have a lower alkoxy group on the phenyl ring thereof; the carbon bond between the 3- and 4-positions of the carbostyril backbone represents a single bond or a double bond] or a salt thereof.

[Effect] The TSC-22 inducer of the present invention is useful in the treatment of osteoporosis and other forms of dysosteogenesis, and the treatment of heart disease and kidney disease, based on the inductive action.

[Scope of Claims]

[Claim 1] A TSC-22 inducer comprising as an active ingredient at least one kind of compound selected from among a carbostyril derivative represented by the general formula [FORMULA 1]:



[wherein R represents a benzoyl group having a lower alkoxy group as a substituent on the phenyl ring thereof; the carbon

bond between the 3- and 4-positions of the carbostyril backbone represents a single bond or a double bond] and a salt thereof.

[Claim 2] The TSC-22 inducer according to claim 1, wherein the carbostyril derivative is 6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-3,4-dihydrocarbostyril.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Belongs] The present invention relates to a TSC-22 inducer whose active ingredient is a particular carbostyril derivative. The TSC-22 inducer of the present invention, based on the action thereof to induce the expression of the TSC-22 gene, is useful in the treatment of osteoporosis and other forms of dysosteogenesis, endocrine disease or heart disease and kidney disease due to water retention secondary thereto. The above-described action is useful in the elucidation of the mechanism of the onset of agranulocytosis as an adverse reaction of the inducer, and is also useful in the development of a method of predicting or preventing the pathogenesis thereof.

[0002]

[Prior Art] 6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-3,4-dihydrocarbostyril (hereinafter also referred to as vesnarinone) and other carbostyril derivatives were developed as oral inotropic agents for use in the treatment of chronic heart disease (J. Am. Coll. Cardio. 9: 865-871 (1987)), and have recently been reported to have anti-proliferation activity and differentiation- and apoptosis-induction activity on some tumor cells, including a human salivary gland cancer cell line (hereinafter also referred to as TYS cells; Am. J. Pathol. 124:496-509 (1986)) in vivo and in vitro (Acta Histochem. Cytochem. 277:591-599 (1994); Cancer Lett. 91:1-9 (1995)). For this reason, in Japan, drugs whose active ingredient is the compound have been under clinical studies as anticancer agents for solid tumors of the head and neck.

[0003] We recently reported that TGF-β1 mRNA and $p21^{waf1}$ mRNA, and proteins thereof, are induced by treating TYS cells with vesnarinone (Cancer Lett. 112:181-189 (1997)).

[0004]

[Problems to Be Solved by the Invention] The present invention is intended to provide a new action of carbostyril derivatives such as vesnarinone, and a novel application based on the action. More specifically, the present invention is intended to provide a novel application for carbostyril derivatives such as vesnarinone or salts thereof as TSC-22 inducers.

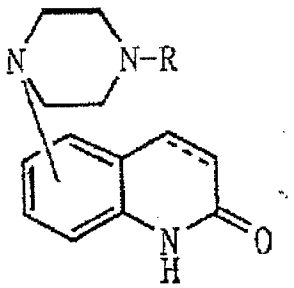
[0005]

[Means of Solving the Problems] The present inventors conducted extensive investigations with the aim of developing a novel application for vesnarinone and other particular carbostyril derivatives, and found that the expression of TSC-22 mRNA is enhanced by treating TYS cells with vesnarinone, and also found that the induced TSC-22 plays important roles in the control of cell actions and cell functions such as cell growth/proliferation and promotion of the signal transduction system in cells based on the regulation of the expression of cytokines such as TGFβ and IFN-γ via CNP (C-type natriuretic peptide) [Eur. J. Biochem., 242, 460-466 (1996)]. The present invention is based on these findings.

[0006] Accordingly, the present invention provides a TSC-22 inducer comprising as an active ingredient at least one kind of compound selected from among a carbostyril derivative represented by the general formula (1) below and a salt thereof.

[0007]

[FORMULA 2]



(1)

The present invention also provides the above-described TSC-22 inducer wherein the carbostyril derivative is 6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-3,4-dihydrocarbostyril.

[0008] The inducer of the present invention, as shown in an Experimental Example below, induces the expression of the above-described desired TSC-22 gene, via production of a protein such as TGF-β1, or directly without being mediated thereby.

[0009] The TSC-22 gene is known to be highly expressed in the brain and the heart, and low expressed in the spleen and the liver, with almost no expression in peripheral blood. The gene has been shown to be induced immediately after treatment of osteoblasts with TGF-β, and to be induced in response to hormones such as FSH. Furthermore, TSC-22 is also known to bind to the promoter sequence of type C natriuretic peptide (CNP) to directly induce the transcription thereof.

[0010] Hence, the TSC-22 inducer of the present invention is useful as a therapeutic and prophylactic drug for various diseases such as osteoporosis and other forms of dysosteogenesis, endocrine disease, or heart disease and kidney disease due to water retention secondary thereto. The TSC-22 inducer of the present invention is also useful in the development of a method of predicting or preventing the expression of hemotoxicity and hepatic/splenic toxicity by various drugs including this inducer.

[0011]

[Modes of Embodiment of the Invention] The groups shown in the general formula (1) above are more specifically exemplified as follows. Examples of the lower alkoxy group include linear or

branched alkoxy groups having 1 to 6 carbon atoms, such as methoxy, ethoxy, propoxy, isopropoxy, butoxy, tert-butoxy, pentyloxy, and hexyloxy groups.

[0012] Examples of the benzoyl group that may have a lower alkoxy group as a substituent on the phenyl ring thereof include benzoyl groups that may have 1 to 3 linear or branched alkoxy groups having 1 to 6 carbon atoms as substituents on the phenyl ring thereof, such as benzoyl, 2-methoxybenzoyl, 3-methoxybenzoyl, 4-methoxybenzoyl, 2-ethoxybenzoyl, 3-ethoxybenzoyl, 4-ethoxybenzoyl, 3-isopropoxybenzoyl, 4-butoxybenzoyl, 2-pentyloxybenzoyl, 3-hexyloxybenzoyl, 3,4-dimethoxybenzoyl, 2,5-dimethoxybenzoyl, and 3,4,5-trimethoxybenzoyl groups.

[0013] Of the above-described carbostyril derivatives used as active ingredients in the present invention, particularly preferred ones include, for example, 6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-3,4-dihydrocarbostyril ("vesnarinone").

[0014] Salts of the above-described carbostyril derivatives include pharmacologically acceptable acid addition salts formed using ordinary acids. Examples of acidic compounds that form such salts specifically include inorganic acids such as sulfuric acid, phosphoric acid, nitric acid, hydrochloric acid, and hydrobromic acid; organic acids such as acetic acid, oxalic acid, maleic acid, fumaric acid, malic acid, tartaric acid, citric acid, succinic acid, ethanesulfonic acid, p-toluenesulfonic acid, and benzoic acid.

[0015] A carbostyril derivative represented by the general formula (1) and a salt thereof can be produced by, for example, the process described in JP-B-HEI-1-43747.

[0016] A carbostyril derivative of the general formula (1) or a salt thereof, being an active ingredient of the inducer of the present invention, is normally used in the form of a common pharmaceutical preparation. Such a preparation is prepared using a diluent or excipient in common use, such as a

filling agent, a bulking agent, binder, moisturizer, disintegrant, surfactant, and lubricant. This pharmaceutical preparation permits the choice of various forms according to the purpose of treatment therewith; representative examples include tablets, pills, powders, solutions, suspensions, emulsions, granules, capsules, suppositories, injections (solutions, suspensions and the like) and the like.

[0017] When the inducer of the present invention is molded in the form of tablets, carriers conventionally commonly known in the art can be widely used; examples include excipients such as lactose, saccharose, sodium chloride, glucose, urea, starch, calcium carbonate, kaolin, crystalline cellulose, and silicic acid; binders such as water, ethanol, propanol, simple syrup, glucose solution, starch solution, gelatin solution, carboxymethylcellulose, shellac, methylcellulose, potassium phosphate, and polyvinylpyrrolidone; disintegrants such as dry starch, sodium alginate, agar powder, laminaran powder, sodium hydrogen carbonate, calcium carbonate, polyoxyethylene sorbitan fatty acid esters, sodium lauryl sulfate, stearic monoglyceride, starch, and lactose; disintegration suppressants such as saccharose, stearin, cacao butter, and hydrogenated oils; absorption promoters such as quaternary ammonium bases and sodium lauryl sulfate; moisturizers such as glycerin and starch; adsorbents such as starch, lactose, kaolin, bentonite, and colloidal silica; lubricants such as purified talc, stearates, boric acid powder, and polyethylene glycol; and the like. Furthermore, tablets can also be prepared as tablets with ordinary coatings applied as required, such as sugar-coated tablets, gelatin-coated tablets, enteric coated tablets, and film-coated tablets, and can also be prepared as double-layer tablets or multiple-layer tablets.

[0018] When the inducer of the present invention is molded in the form of pills, carriers conventionally commonly known in the art can be widely used; examples include excipients such as glucose, lactose, starch, cacao butter, hardened vegetable

oils, kaolin, and talc; binders such as gum arabic powder, tragacanth powder, gelatin, and ethanol; disintegrants such as laminaran agar; and the like.

[0019] When the inducer of the present invention is molded in the form of suppositories, carriers conventionally commonly known in the art can be widely used; examples include polyethylene glycol, cacao butter, higher alcohols, esters of higher alcohols, gelatin, semi-synthetic glycerides and the like.

[0020] When prepared as injections, solutions and suspensions are preferably sterilized and isotonic to the blood; when the inducer of the present invention is molded in the form of these solutions, emulsions and suspensions, all diluents in common use in the art can be used; examples include water, ethyl alcohol, propylene glycol, ethoxidated isostearyl alcohol, polyoxydated isostearyl alcohol, polyoxyethylene sorbitan fatty acid esters and the like. In this case, sodium chloride, glucose, glycerine or the like in an amount sufficient to prepare an isotonic solution may be contained in the pharmaceutical preparation, and ordinary solubilizers, buffering agents, soothing agents and the like may be added.

[0021] Furthermore, in the inducer of the present invention, there may be contained as required a coloring agent, a preservative, a flavoring agent, a taste corrective, a sweetening agent and the like and other pharmaceutical products.

[0022] The amount of a carbostyril derivative represented by the formula (1) or a salt thereof contained as an active ingredient in the inducer of the present invention is not particularly limited, and is chosen as appropriate from a broad range; it is appropriate that the amount be usually in the range of about 1 to 70% by weight, preferably about 1 to 30% by weight, in the entire composition.

[0023] The method of administration of the thus-obtained inducer of the present invention is not particularly limited,

and is determined according to the form of the preparation, the patient's age, sex and other conditions, and the severity of the disease. For example, when the inducer of the present invention is prepared as a pharmaceutical preparation in the form of an injection, it can be administered by intravenous, intramuscular, subcutaneous, intradermal, and intraperitoneal administration and the like, and this can also be administered intravenously after being mixed as required with an ordinary infusion such as glucose and amino acids. When the inducer of the present invention is prepared as a pharmaceutical preparation in the form of a solid preparation such as tablets, pills, granules, and capsules, or in the form of a solution for oral administration, it can be administered orally or intestinally. Suppositories can be administered intrarectally.

[0024] The dose of the inducer of the present invention can be chosen as appropriate from a broad range, without particular limitations; it is usually recommended that a carbostyril derivative of the general formula (1) or a salt thereof be administered at a daily dose per kg body weight chosen from the range of about 0.5 to 30 mg, and it is appropriate that about 10 to 1000 mg of these active ingredients be contained in a unit dosage form. The TSC-22 inducer of the present invention can be administered in a single dose in a day or in 3 to 4 divided doses in a day.

[0025]

[Examples] Hereinafter, preparation examples and experimental examples are given to describe the present invention in further detail.

[0026]

[Preparation Example 1]

Vesnarinone	5 mg
Starch	132 mg
Magnesium stearate	18 mg
Lactose	45 mg
Total	200 mg

Tablets containing the above composition per tablet were produced.

[0027]

[Preparation Example 2]

Vesnarinone	150 g
Avicel (trade name, produced by Asahi Kasei Corporation)	40 g
Cornstarch	30 g
Magnesium stearate	2 g
Hydroxypropylmethylcellulose	10 g
Polyethylene glycol-6000	3 g
Castor oil	40 g
Methanol	40 g

After the above active ingredient compound, Avicel, cornstarch and magnesium stearate were blended and polished, the blend was tableted using pounders for R10 mm sugar-coated tablets. The tablets obtained were coated with a film-coating agent consisting of hydroxypropylmethylcellulose, polyethylene glycol-6000, castor oil and methanol to yield film-coated tablets.

[0028]

[Preparation Example 3]

Vesnarinone	150.0 g
Citric acid	1.0 g
Lactose	33.5 g
Dicalcium phosphate	70.0 g
Pluronic F-68	30.0 g
Sodium lauryl sulfate	15.0 g
Polyvinylpyrrolidone	15.0 g
Polyethylene glycol (Carbowax 1500)	4.5 g
Polyethylene glycol (Carbowax 6000)	45.0 g
Cornstarch	30.0 g
Dry sodium lauryl sulfate	3.0 g
Dry magnesium stearate	3.0 g
Ethanol	appropriate amount

The above active ingredient compound, citric acid, lactose,

dicalcium phosphate, Pluronic F-68 and sodium lauryl sulfate were blended.

[0029] This blend was sieved through a No.60 screen, and wet-granulated with an alcoholic solution containing polyvinylpyrrolidone, Carbowax 1500 and Carbowax 6000. With alcohol added as required, the powder was made into a pasty mass. Cornstarch was added, and blending was continued until uniform particles were formed. The particles were passed through a No.10 screen, placed in a tray, and dried in a 100°C oven for 12 to 14 hours. The dry particles were sieved through a No.16 screen, dry sodium lauryl sulfate and dry magnesium stearate were added, and the ingredients were blended and compression-molded into a desired shape using a tableting machine.

[0030] The above-described core was treated with varnish, and talc was spread to prevent moisture absorption. Coated around the core was an undercoat. Varnish was coated sufficient times to allow oral ingestion. To make the tablets completely round and smooth, an undercoat and a smooth coat were further applied. Color coating was performed until the desired color was obtained. After drying, the coated tablets were polished to prepare tablets with uniform gloss.

[0031]

[Experimental Examples] Preparation of the vesnarinone and TYS cells used in the Experimental Examples given below, and the various operations used in the Experimental Examples were performed as directed below.

[0032] (1) Preparation of vesnarinone solution

Vesnarinone was previously prepared as a stock solution at a 10 mg/ml concentration using dimethylsulfoxide (DMSO). The anti-proliferation activity of vesnarinone in the stock solution was stable at 4°C for at least 1 month. The stock solution was diluted with a complete medium to obtain a desired concentration before use.

[0033] (2) Preparation and cultivation of TYS cells: Human

salivary gland squamous cell carcinoma cell TYS [Am. J. Pathol., 124, 496-509 (1986)] was prepared by cultivation using Dulbecco's modified Eagle medium (DMEM; Gibco Labs.) supplemented with 10% fetal calf serum (FCS; Bio-Whittaker), 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco Labs.) and 0.25 μg/ml amphotericin B (Gibco Labs.), in the presence of 95% air and 5% CO_2, in a humid atmosphere at 37°C.

[0034] (3) Polymerase chain reaction (PCR)

PCR was performed using a thermal sequencer (Iwaki Glass) with the final dNTPs concentration and final primer concentration in the reaction liquid being 200 μM and 1 μM, respectively, and with Taq DNA polymerase (Takara) added to the reaction liquid to obtain a final concentration of 0.05 U/μl (94°C for 3 minutes, 94°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 2.5 minutes in 30 cycles, followed by an extension of 72°C for 4 minutes).

(4) Northern blot analysis

Cytoplasmic RNA (20 μg) was electrophoresed on formaldehyde/1.0% agarose gel, and this was blotted onto a nylon filter (Hybond-N^+; Amersham). This nylon filter was hybridized to a ^{32}P-labeled cDNA probe in the presence of 50% formamide, 5×physiological saline-sodium phosphate-EDTA (saline-sodium phosphate-EDTA), 0.1% SDS, 5×Denhardt's solution and 100 μg/ml salmon sperm DNA at 42°C for 15 to 20 hours. The filter was thoroughly washed with 0.1×SSC (standard saline citrate) - 0.5% SDS twice at room temperature and once at 50°C for 40 minutes. Exposure to X-ray films was performed using a contrast enhancement screen at -70°C.

[0035] The probes used were a 3' terminal portion (1.2 kb) of human TSC-22 cDNA, a 875-bp fragment of human $p21^{waf1}$ cDNA having a complete open reading frame, cloned from TYS cells (Cancer Lett. 112: 181-189 (1997)), and an Xho1-Xho1 fragment (2.1 kb) of human β-actin pHFβA-1 (ATCC No.77644:HFBCA46).

[0036] Densitometric analysis of signals was performed using the NIH image 1.44 program and/or the BAS-2000II image

analysis system (Fuji photo film).

[0037] Experimental Example 1 Influence of vesnarinone on in vitro cell proliferation

The influence of vesnarinone on the in vitro proliferation of TYS cells (anti-proliferation action) was evaluated using the MTT assay method (3-(3,4-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Cancer Res. 47: 936-942 (1987)).

[0038] Specifically, TYS cells were sown into a DMEM containing 10% FCS on a 96-well plate (Falcon; Becton Dickinson Labware) at 2×10^3 cells/well. After 24 hours, the cells were transferred to DMEM (containing 10% FCS) media containing various concentrations of vesnarinone (0, 0.1, 1, 10, 50 μg/ml); after 2 to 4 days, the number of cells was determined by an assay using MTT (Sigma).

[0039] The results are shown in FIG. 1. As seen therefrom, vesnarinone minimally suppressed the proliferation of the TYS cells at concentrations of 0.1 μg/ml to 10 μg/ml, but remarkably suppressed the same at a concentration of 50 μg/ml. The proliferation inhibitory activity of vesnarinone on the TYS cells was cytostatic but not cytocidal. The TYS cells treated with 50 μg/ml vesnarinone dilated and ceased their cell division, but did not detach from the bottom of the culture dish.

[0040] The anti-proliferation action of the DMSO used as a solvent for preparing vesnarinone was checked. As a result, the DMSO slightly inhibited the proliferation of the TYS cells, but the extent thereof was much lower than the anti-proliferation action of vesnarinone.

[0041] Experimental Example 2 Cell cycle analysis

TYS cells were cultured in the presence or absence of vesnarinone (50 μg/ml) for 24 hours, 48 hours and 72 hours, and then collected in a conical tube (Falcon); the cells were fixed with 70% ethanol, and washed with phosphate-buffered physiological saline. After treatment with 100 μg/ml RNaseA (Sigma), the cells were stained with 40 μg/ml propidium iodide

(Molecular probes); next, the cell cycle was analyzed using the digitized flowcytometry system EPICS (Coulter).

[0042] 50% of the TYS cells in the vesnarinone-untreated group exhibited the S phase and G2/M stage 24 hours after medium exchange. However, after 48 hours and 72 hours, the ratio of cells exhibiting these stages decreased gradually (FIG. 2 and Table 1). This is probably attributable to proliferation suppression due to exhaustion of, and/or inhibition of contact, with the growth factor in the medium. On the other hand, in the vesnarinone-treated group, the ratio of cells exhibiting the S phase and G2/M stage 24 hours after addition of the drug was much lower than that for the above-described untreated group (FIG. 2 and Table 1). When exposed to vesnarinone for 48 hours or more, most TYS cells ceased their cell cycle at the G1 stage (FIG. 2 and Table 1).

[0043]

[Table 1]

	Untreated TYS cells			Treated TYS cells		
	24h	48h	72h	24h	48h	72h
G0/G1	50.5%	56.5%	72.7%	76.2%	81.8%	92.0%
S	24.6%	17.6%	11.1%	10.3%	7.2%	2.0%
G2/M	20.3%	21.7%	13.3%	11.0%	6.4%	4.7%

From these findings, it is seen that vesnarinone suppresses cell proliferation by inducing G1 arrest.

[0044] Experimental Example 3 Isolation of human TSC-22 cDNA

TSC-22 cDNA as a vesnarinone induction gene was isolated from a cDNA library constructed from TYS cells treated with vesnarinone. Specifically, this was performed as described below.

[0045] (1) Isolation of RNA

TYS cells were treated with vesnarinone (50 μg/ml) for 3 days. Next, the cells obtained were lysed with a hypotonic buffer solution containing Nonidet p-40 (Sigma), and the nuclei were removed, to yield total cytoplasmic RNA. The total cytoplasmic

RNA was subjected to oligo(dT)-cellulose chromatography in two cycles to prepare Poly(A)$^+$ RNA.

[0046] (2) Construction of cDNA library

5 μg of the Poly(A)$^+$ RNA obtained was reverse-transcribed by M-MLV (Gibco) using an oligo(dT)-XhoI primer/linker (Stratagene). A second-strand cDNA was synthesized using a cDNA synthesis kit (Stratagene), and an EcoRI adapter was ligated as directed in the manual of the kit. After the synthetic cDNA was cleaved with XhoI, it was ligated to the cloning vector ZAP Express (Stratagene), previously digested with EcoRI and SalI. The cDNA was inserted in the antisense-orientation by a promoter of eukaryotic cells and prokaryotic cells in the vector. The primary cDNA library contained about 1.5×10^5 clones, 90% of which were recombinants. The ZAP Express library was used after one cycle of amplification.

[0047] (3) Random sequencing

From the above-described cDNA library constructed from the vesnarinone-treated TYS cells, 10^7 clones were randomly sequenced.

[0048] Specifically, a randomly selected pBK-CMV-library-transformant colony was picked up using a toothpick, and cultured in 6 ml of an LB medium containing 50 μg/ml kanamycin overnight. Phagemid was extracted by the alkali lysis method, half of the phagemid was digested with EcoRI and PstI, and the cDNA inserts were cut out. Most of the inserts ranged from 0.5 to 2 kb in length. The inserts were purified from agarose gel using Gene Clean Kit II (Bio 101), and used as probes for Northern blot analysis.

[0049] The remaining half of the phagemid was subjected to sequencing analysis. The cDNA sequences were examined by the dideoxy chain termination method using FITC-labeled primers and the Takara Taq Cycle Sequencing Kit or the Amersham Thermo Sequenase Cycle Sequencing Kit (Amersham). Electrophoresis and scanning were performed using the Shimadzu DSQ-500 DNA sequencer (Shimadzu Corporation). About 200 to 300 base pairs

of DNA sequences were detected in individual clones. All nucleic acids were subjected to homology search through the Gene Bank or EMBL database using the BLAST program.

[0050] As a result, about 64% of the clones were identified as commonly known genes, 18% as functionally unknown genes registered in a database, and 18% as unknown genes.

[0051] In the commonly known genes, the ratio of housekeeping genes of the vesnarinone-treated TYS library was lower than that of the cDNA library constructed in an ordinary way (vesnarinone-untreated). From this finding, vesnarinone treatment was found to reduce the expression of housekeeping genes, and to relatively increase the expression of genes related to cell proliferation, apoptosis and differentiation in TYS cells. About 30% of the commonly known genes (for example, TSC-22, HSC70, CK19, SOS, NGFR-related lymphocyte activating molecule, IL-6, TAFIIA, elongation factor-1γ, IL-1/TNF induction EST, DNA binding protein A, annexin II, DNA-dependent protein kinase catalyst subunit, rab-GDI, SUII translation initiation factor, lysosome protection protein, TNF-α induction protein B12, ADP-ribosylation factor, phospholipase A2 and 13 kDa differentiation-related protein and the like) seem to be related to cell proliferation, differentiation and apoptosis.

[0052] The expression of the above-described commonly known genes and unknown genes was compared between vesnarinone-untreated TYS cells and vesnarinone-treated TYS cells using Northern blotting. As a result, it was demonstrated that the expression of TSC-22 mRNA is increased by vesnarinone treatment. However, the expression of other genes remained unchanged, or decreased, by treatment of TYS cells with vesnarinone.

[0053] (4) Isolation of human TSC-22 cDNA

Since a clone obtained by random sequencing (3′ terminus of pBK-CMV-hTSC-22) contained a 1313-bp human TSC-22 cDNA fragment only, and did not contain a complete open reading

frame, cloning of the full-length human TSC-22 cDNA was attempted.

[0054] 100 ng of a pBK-CMV library containing vesnarinone-treated TYS cDNA in an antisense orientation form CMV promoter was amplified by PCR using the DP1 primer.

[0055] Next, a T7 RNA polymerase promoter lying upstream of the cDNA was inserted into a vector. Subsequently, the PCR product was subjected to a second round of PCR. For the second-round PCR, used as primers were the DP2 primer lying 7 bp upstream of the DP1 primer, and comprising a PstI site, and mUP acquired from the mouse or rat TSC-22 sequence lying 92 bp upstream from the mouse TSC-22 translation initiation codon or lying 84 bp upstream in the rat (J. Biol. Chem. 267:10219-10224 (1992); Endocrinology 134: 1205-1212 (1994)).

[0056] The second-round PCR product containing the 5′ terminus of human TSC-22 was subcloned into the pUC19 vector (5′ terminus of pUC19-hTSC-22). A 3′-terminal fragment of PstI-digested hTSC-22, derived from the 3′ terminus of pBK-CMV-hTSC-22, was ligated to the 5′ terminus of pUC19-hTSC-22, previously digested with PstI. Nearly full-length human TSC-22-cDNA was isolated therefrom.

[0057] The base sequences of the various primers and promoter used are shown in Table 2.

[0058]

[Table 2]

Primers	DP1 : 5'-agccagtctgcagctgggcctgaa-3'	
	DP2 : 5'-tctgcagctgggcctgaaactgggc-3'	
	mUP : 5'-atctagtttgaaccaggctg-3'	
T7 RNA polymerase promoter	: 5'-taatacgactcactataggg-3'	

The nucleotide sequence of the human TSC-22 acquired from the salivary gland cancer cell line in the present invention agreed completely with that recently acquired from a human embryo by Joy et al.(Biochem. Biophys. Res. Commun. 222: 821-

826 (1996)). Nucleotide sequence analysis showed that the human TSC-22 gene has an overall homology of 79% to the mouse or rat TSC-22 gene. However, the nucleotide sequence in the human TSC-22 coding region showed a 92% agreement with that of mouse or rat TSC-22.

[0059] Meanwhile, as deduced therefrom, the amino acid sequence of human TSC-22 protein showed a 98% agreement with that of mouse or rat TSC-22 protein. Mouse and rat TSC-22 protein contained 143 amino acids (Shibanuma et al., J. Biol. Chem., 267, 10219-10224 (1992); Hamil and Hall, Endocrinology 134, 1205-1212 (1994)), whereas human TSC-22 protein contained 144 amino acids. Additionally, in human TSC-22, one serine residue had been inserted at the codon 43-position, and in the mouse and rat, the serine residue at the codon 141-position (in humans, codon 142-position) had been substituted by a proline residue. Also, human TSC-22 protein was found to conserve a leucine zipper-like structure in the region between leucine-77 and leucine-98.

[0060] Note that TSC-22 protein is a protein having a leucine zipper-like structure, and has been reported to act as a TGF-β or FSH inducible transcription regulator (TGF-β or FSH inducible putative transcriptional regulator).

[0061] Experimental Example 4 Induction of expression of TSC-22 mRNA by treatment of TYS cells with vesnarinone

(1) The expression of TSC-22 mRNA induced by treating TYS cells with vesnarinone was examined using Northern blot analysis.

[0062] Specifically, TYS cells were treated with vesnarinone (50 μg/ml DMEM); next, cytoplasmic RNA was prepared from the TYS cells, and electrophoresed on 1% denatured agarose gel. This was transferred onto a nylon filter, and hybridized with ^{32}P-labeled probes for human TSC-22 and β-actin, and the status of expression of TSC-22 mRNA was examined by autoradiography. The autoradiograph obtained was evaluated using a densitometric scanner. For a control experiment, TYS cells not

treated with vesnarinone were similarly examined for the expression of TSC-22 mRNA. The results are shown in FIG. 3.

[0063] In the TYS cells, about 1.8-kb TSC-22 mRNA was detected. The TSC-22 mRNA level in the TYS cells increased persistently until the cells reached a confluence (FIG. 3). The expression of TSC-22 mRNA in the TYS cells was remarkably increased by 50 μg/ml vesnarinone treatment (day 1: 225% of control, day 2: 164% of control, day 3: 125% of control) (FIG. 3, B). The induction of TSC-22 mRNA persisted for at least 3 days after addition of vesnarinone.

[0064] (2) Next, the status of induction of TSC-22 mRNA by vesnarinone treatment was monitored over time. For reference, the same was performed using transforming growth factor-β1 (TGF-β1), a commonly known TSC-22 induction factor. Specifically, TYS cells were treated with each of vesnarinone and TGF-β1 for times of 1, 2, 6, 12 and 24 hours, cytoplasmic RNA was prepared from the TYS cells obtained, and the RNA (20 μg/lane) was electrophoresed on 1% denatured agarose gel. Next, the RNA was transferred onto a nylon filter, hybridized with ^{32}P-labeled probes for human TSC-22 and β-actin, and subjected to autoradiography. The autoradiograph obtained was evaluated using a densitometric scanner. The results are shown in FIG. 4.

[0065] As seen therefrom, vesnarinone treatment for a short time did not induce TSC-22 mRNA so much, but by treatment for 24 hours, the expression of TSC-22 mRNA was increased rapidly (FIG. 4, B). On the other hand, by TGF-β1 treatment, TSC-22 mRNA was induced quickly in short times (FIG. 4, B).

[0066] (3) To determine whether or not the induction of expression of TSC-22 mRNA is a direct effect of vesnarinone, TYS cells were treated with cycloheximide, a protein synthesis inhibitor, and its influence on the gene induction was examined. For reference, the influence of cycloheximide on the expression of $p21^{waf1}$ mRNA induced by vesnarinone was also examined.

[0067] Specifically, TYS cells were treated in the presence or

absence of 10 μg/ml cycloheximide for 2 hours, 4 hours or 6 hours with vesnarinone (50 μg/ml), and cytoplasmic RNA was prepared from the TYS cells obtained, and electrophoresed on 1% denatured agarose gel (RNA: 20 μg/lane). Next, the RNA was transferred onto a nylon filter, hybridized with ^{32}P-labeled probes for human TSC-22, p21^{waf1} and β-actin, and subjected to autoradiography. The autoradiograph obtained was evaluated using a densitometric scanner. For control, TYS cells not treated with vesnarinone were subjected to the same experiment. The results are shown in FIG. 5.

[0068] As seen therefrom, by treatment with 10 μg/ml cycloheximide, probably as a result of the accumulation of mRNA, the expression of TSC-22 mRNA was increased remarkably, but the induction of the TSC-22 gene by vesnarinone was suppressed (FIG. 5, B). Meanwhile, the induction of the p21^{waf1} gene by vesnarinone was not inhibited by treatment with cycloheximide (FIG. 5).

[0069] (4) Conclusion

As described above, the expression of TSC-22 mRNA was increased slightly by treatment with vesnarinone for several hours, but remarkable induction of TSC-22 mRNA was observed 24 hours after addition of vesnarinone, and it persisted for at least 3 days. On the other hand, quick induction of TSC-22 mRNA was observed in a short time of TGF-β1 treatment. Shibanuma et al. and Hamil and Hall reported that the induction of mouse and rat cell TSC-22 by TGF-β1 or FSH, like Jun and Fos, was rapid but transient (J. Biol. Chem. 267:10219-10224 (1992); Endocrinology 134: 1205-1212 (1994)). We previously reported that vesnarinone promoted the induction of TGF-β1 protein in TYS cells (Cancer Lett. 112: 181-189 (1997)).

[0070] From these facts, it is thought that at least in the human TYS cell system of the present invention, vesnarinone first induces the expression of TSC-22 mRNA via the direct action thereof, and then acts to enhance the expression of

TSC-22 mRNA via production of other proteins such as TGF-β.

[0071] Experimental Example 5 Detection of TSC-22 protein

(1) 100 μg of protein prepared from TYS cells was subjected to SDS-PAGE. The protein was transferred from the gel onto a nitrocellulose filter (Bio-Rad). The TSC-22 protein on the membrane was detected using an affinity-purified anti-glutathione-S-transferase (GST)-TSC-22 fusion protein rabbit polyclonal antibody and the Amersham ECL kit (Amersham). The results are shown in FIG. 6A. As seen from the figure, two bands were detected at the 20 kDa and 18 kDa positions. The slower band seems to be a phosphorylated form of TSC-22 protein.

[0072] (2) The increase in the expression of TSC-22 protein by vesnarinone treatment in TYS cells was determined by solid phase ELISA. 100 μg of the protein was added to a 96-well plate (Falcon), and the plate was incubated at room temperature for 3 hours. A primary antibody (affinity purified anti-GST-TSC-22 fusion protein rabbit antibody; 1:2000) was added to the wells, and the plate was incubated at room temperature for 3 hours. After the plate was washed with phosphate-buffered physiological saline, HRR-conjugate-sheep anti-rabbit IgG (Amersham, 1:500) was added to the wells, and the plate was incubated at room temperature for 1 hour. Next, 100 μl of TMB (3,3',5,5'-tetramethylbenzidine, 0.1 mg/ml; Sigma) solution was added to the wells, and the plate was incubated for 10 minutes. The reaction was stopped by the addition of 40 μl of 1M H_2SO_4, and absorbance at 450 nm was determined. The results are shown in FIG. 6B.

[0073] By this solid phase ELISA method, it was shown that the amount of TSC-22 protein produced in the TYS cells by vesnarinone treatment increased compared to the untreated cells.

[0074] Experimental Example 6 Influence of antisense oligonucleotide against human TSC-22 mRNA on vesnarinone-treated TYS cells or untreated TYS cells

Antisense phosphorothioate-oligonucleotide against human TSC-22 mRNA and sense phosphorothioate-oligonucleotide were synthesized (Table 3).

[0075]

[Table 3]

Antisense phosphorothioate-oligonucleotide	: 5'-tgggatttCATgcaattgca-3'
Sense phosphorothioate-oligonucleotide	: 5'-tgcaattgcATGaaatccca-3'

Vesnarinone-treated TYS cells and untreated TYS cells were separately treated with these oligonucleotides, and their influences on cell proliferation were examined. Specifically, the cells were treated by adding these oligonucleotides directly to the cultured cells when the TYS cells reached a confluence or the cells proliferated rapidly. The number of cells was evaluated by MTT assay.

[0076] The results are shown in FIG. 7. As seen therefrom, when the TYS cells were treated with 10 μM of the sense oligonucleotide, probably as a result of the non-specific cytotoxicity of the high-dose of the oligonucleotide, cell proliferation was inhibited slightly. However, treatment with the same concentration of the antisense oligonucleotide promoted the proliferation of the TYS cells ($p<0.01$). Furthermore, the antisense oligonucleotide suppressed the anti-proliferation action of vesnarinone on TYS cells. However, because treatment of TYS cells with vesnarinone was begun when the cells reached a confluence in this experiment, the anti-proliferation effect of vesnarinone on TYS cells was not as intense as that shown in FIG. 1.

[0077] Meanwhile, when TYS cells were treated with the antisense oligonucleotide under low-density culture conditions (2×10^3/well) in the presence or absence of vesnarinone, no clear effect of the antisense oligonucleotide could be shown. As shown in FIG. 3, the expression of TSC-22 mRNA in the TYS cells was very low under the low-density culture conditions, but it increased gradually until the cells reached a

confluence; from this finding, the antisense oligonucleotide is thought to have effectively inhibited the expression of TSC-22 mRNA in the TYS cells under the high-density culture conditions.

[0078] The above-described experiments lead to the discussion below.

[0079] The expression level of TSC-22 mRNA was well proportional to culture cell density and cells cycle. Although the level of TSC-22 mRNA was low in the proliferation environment, the mRNA level rose remarkably when the cells reached a confluence. Vesnarinone increased the expression of TSC-22 mRNA in TYS cells and remarkably suppressed cell proliferation by terminating the cell cycle in the G1 phase. The antisense oligonucleotide against TSC-22 mRNA promoted the proliferation of TYS cells and suppressed the anti-proliferation effect of vesnarinone after the cells reached a confluence. From these findings, TSC-22 seems to negatively control the growth/proliferation of the TYS cell line, and to mediate the proliferation inhibitory signal from vesnarinone in TYS cells at least partially.

[0080] The amino acid sequence of human TSC-22 protein showed a 98% agreement with the mouse and rat sequences (1 amino acid inserted and 1 amino acid substituted). This fact of the high degree of conservation of the amino acid sequence across the different species demonstrates that TSC-22 plays an important role in the control of cell actions such as cell growth/proliferation, differentiation and apoptosis.

[0081] Shibanuma et al. and Hamil and Hall reported that human TSC-22 protein lacks the N-terminal basic region of the leucine zipper domain (J. Biol. Chem. 267:10219-10224(1992); Endocrinology 134: 1205-1212 (1994)). From this fact, human TSC-22, like CHOP (Genes Dev. 6:439-453 (1992)) or IP-1 (Cell 64: 983-993 (1991)), is thought to interact with a basic leucine zipper transcriptional factor to act as a dominant negative control factor. CHOP is homologous to the C/EBP

family of transcriptional factors, is also known as GADD (Growth Arrest and DNA Damage) 153 (Mol. Cell. Biol. 9: 4196-4203 (1989)), and acts as a dominant negative inhibitor of C/EBP (Genes Dev. 6: 439-453 (1992); Genes Dev. 8:453-464 (1994)). The expression of the CHOP (GADD153) gene is induced by some reagents that cause growth suppression or DNA damage (Mol. Cell. Biol. 9: 4196-4203 (1989)). Additionally, the dominant regulator Id is a helix-loop-helix protein lacking a DNA-binding region, and has been well studied with respect to the control of cell growth and differentiation (Cell 61:49-59 (1990)). Ids (Id1, Id2, Id3 and Id4) bind specifically to helix-loop-helix proteins (MyoD, E2A, E12 and E47) to inhibit the DNA binding potentials thereof (Nucleic Acids Research 22:749-755 (1994)). Ids control differentiation in some cell systems, including musculogenesis (Cell 61:49-59 (1990); J. Biol. Chem. 269:6031-6039 (1994)), neurogenesis (Biochem. Biophys. Res. Commun. 199:1355-1362 (1994)) and hemogenesis (Cell 79:893-900 (1994)) and cell growth/proliferation (Proc. Natl. Acad. Sci. USA 91:4985-4988 (1994)).

[0082] Hence, TSC-22 is likely to be a dominant negative control factor like these molecules, and is thought to influence various biological phenomena by inhibiting the functions of many leucine zipper transcriptional factors. The TSC-22 inducer of the present invention is useful for the treatment/prophylaxis of diseases mediated by the target protein thereof.

[0083]

[SEQUENCE LISTING]

SEQ ID NO:1

Sequence length : 24

Sequence type : nucleic acid

Number of chain : single strand

Topology : liner

Sequence variety : other nucleic acid synthetic DNA

Characteristic of sequence

Symbol representing characteristic : primer

Sequence

AGCCAGTCTG CAGCTGGGCC TGAA 24

SEQ ID NO : 2

Sequence length : 25

Sequence type : nucleic acid

Number of chain : single strand

Topology : liner

Sequence variety : other nucleic acid synthetic DNA

Characteristic of sequence

Symbol representing characteristic : primer

Sequence

TCTGCAGCTG GGCCTGAAAC TGGGC 25

SEQ ID NO : 3

Sequence length : 20

Sequence type : nucleic acid

Number of chain : single strand

Topology : liner

Sequence variety : other nucleic acid synthetic DNA

Characteristic of sequence

Symbol representing characteristic : primer

Sequence

ATCTAGTTTG AACCAGGCTG 20

SEQ ID NO : 4

Sequence length : 20

Sequence type : nucleic acid

Number of chain : single strand

Topology : liner

Sequence variety : other nucleic acid synthetic DNA

Characteristic of sequence

Symbol representing characteristic : promoter

Sequence

TAATACGACT CACTATAGGG 20

SEQ ID NO : 5

Sequence length : 20

Sequence type : nucleic acid

Number of chain : single strand

Topology : liner

Sequence variety : other nucleic acid synthetic DNA

Characteristic of sequence

Symbol representing characteristic : modified base

Sequence

TGGGATTTCA TGCAATTGCA 20

SEQ ID NO : 6

Sequence length : 20

Sequence type : nucleic acid

Number of chain : single strand

Topology : liner

Sequence variety : other nucleic acid synthetic DNA

Characteristic of sequence

Symbol representing characteristic : modified base

Sequence

TGCAATTGCA TGAAATCCCA 20

[Brief Description of the Drawings]

[FIG. 1] A graph showing the influence of vesnarinone on the in vitro proliferation of TYS cells. The values are shown as mean values for six measurements. *p<0.01 ** vesnarinone concentration in the medium (μg/ml).

[FIG. 2] A graph showing a cell cycle analysis of TYS cells. Herein A shows the results obtained when TYS cells were cultured in the absence of vesnarinone for 24 hours, 48 hours and 72 hours; B shows the results obtained when TYS cells were

cultured in the presence of 50 μg/ml vesnarinone for 24 hours, 48 hours and 72 hours.

[FIG. 3] A graph showing the influence of vesnarinone on the expression of TSC-22 mRNA in TYS cells. A is a substitute photograph for a graph showing the results obtained by preparing cytoplasmic RNA from vesnarinone (50 μg/ml)-treated TYS cells (+) or untreated TYS cells (-), fractionating it on 1% denatured agarose gel (RNA: 20 μg/lane), transferring it onto a nylon membrane, and hybridizing it with ^{32}P-labeled probes for human TSC-22 and β-actin. B shows the results obtained when the autoradiograph shown in A was analyzed by densitometric scanning.

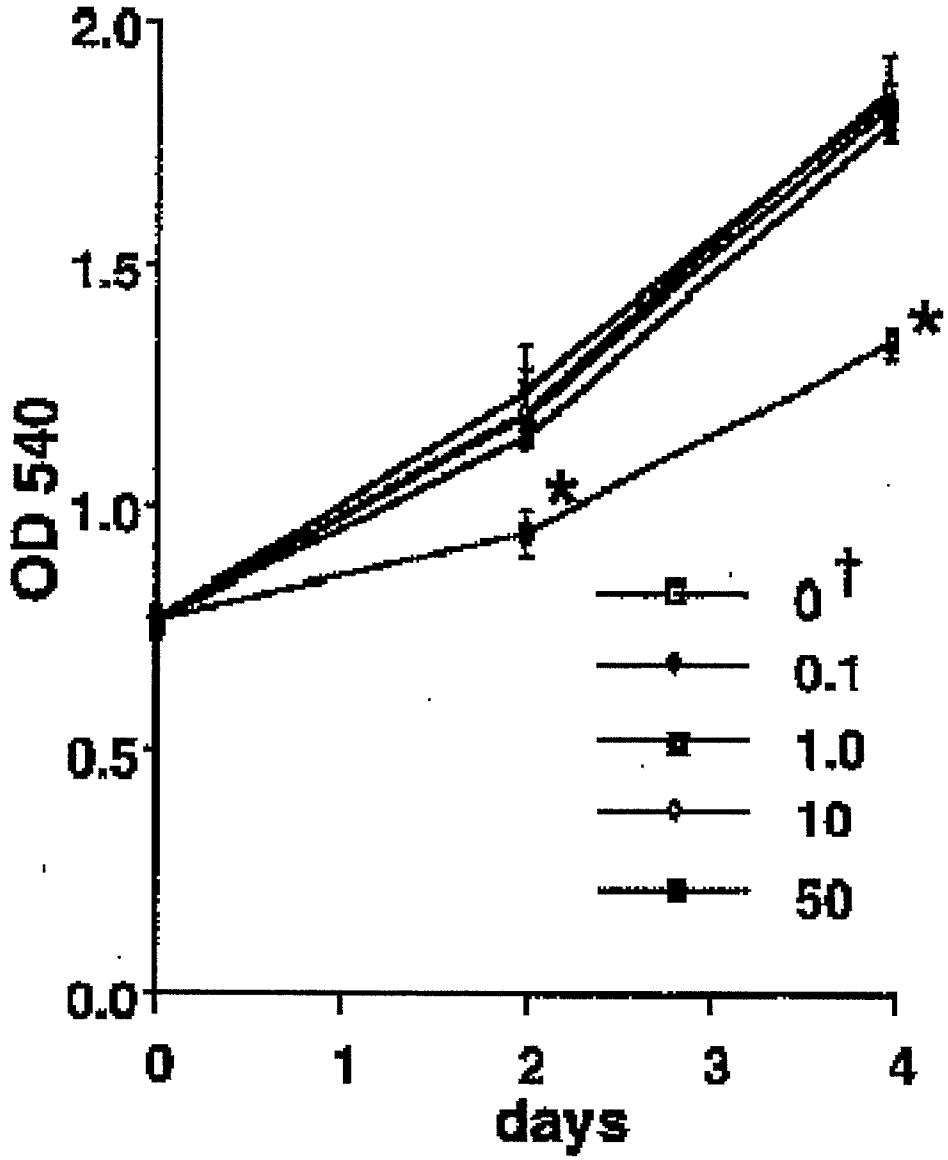
[FIG. 4] A graph showing changes over short time in the induction of TSC-22 mRNA by vesnarinone and TGF-β1 in TYS cells. A is a substitute photograph for a graph showing the results obtained by preparing cytoplasmic RNA from TYS cells treated with vesnarinone or TGF-β1 for the times specified herein (1, 2, 6, 12, 24 hours), fractionating the RNA (20 μg/lane) on 1% denatured agarose gel, transferring it onto a nylon membrane, and then hybridizing it with ^{32}P-labeled probes for human TSC-22 and β-actin. B shows the results obtained when the autoradiograph shown in A was analyzed by densitometric scanning.

[FIG. 5] A graph showing the influence of cycloheximide on the induction of TSC-22 and $p21^{waf1}$ in TYS cells. A is a substitute photograph for a graph showing the results obtained by preparing cytoplasmic RNA from TYS cells treated with vesnarinone (50 μg/ml) for the times specified herein (2, 4, 6 hours) (+) or untreated TYS cells (-) in the presence or absence of 10 μg/ml cycloheximide, fractionating it on 1% denatured agarose gel (RNA: 20 μg/lane), transferring it onto a nylon membrane, and hybridizing it with ^{32}P-labeled probes for human TSC-22, $p21^{waf1}$ and β-actin. B shows the results obtained when the autoradiograph shown in A was analyzed by densitometric scanning.

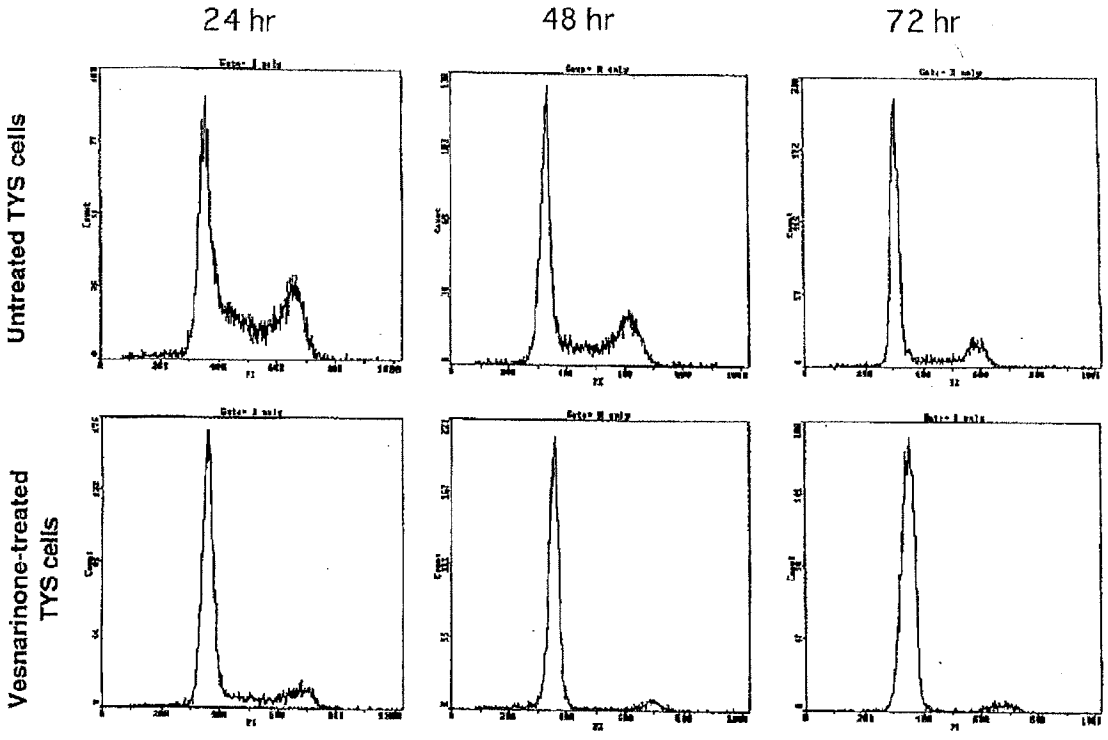
[FIG. 6] A is a substitute photograph for a graph showing the results obtained by detecting TSC-22 protein in TYS cells by Western blotting. A 100-μg protein sample derived from TYS cells (TYS) or 100 ng of purified recombinant GST-TSC-22 protein (GST-TSC-22) was subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and stained with affinity purified anti-GST-TSC-22 antibody. B shows the results of detection of TSC-22 protein in the cells by the solid phase ELISA method. Herein, control shows the results from 100 μg of a protein prepared from untreated TYS cells, DMSO shows the results from 100 μg of a protein prepared from TYS cells treated with 0.5% DMSO for 48 hours, and Ves shows the results from 100 μg of a protein prepared from TYS cells treated with 50 μg/ml vesnarinone for 48 hours. The values are shown as mean values for two measurements. The window chart herein shows a standard curve for solid phase ELISA using the GST-TSC-22 fusion protein as the antigen.

[FIG. 7] A graph showing the influence of an antisense oligonucleotide against human TSC-22 mRNA on vesnarinone-treated or -untreated TYS cells. The numerical values are shown as mean values for six tests.

[FIG. 1]



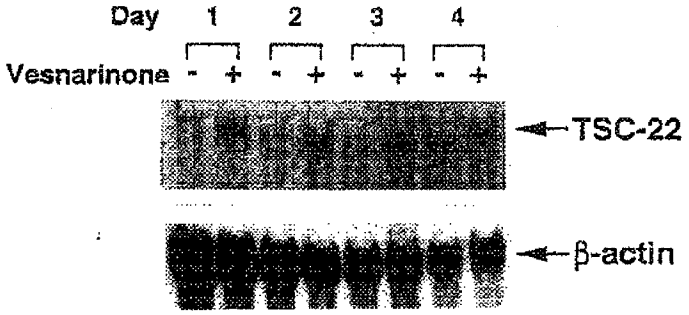
[FIG. 2]


24 hr
48 hr
72 hr
Untreated TYS cells
Vesnarinone-treated TYS cells

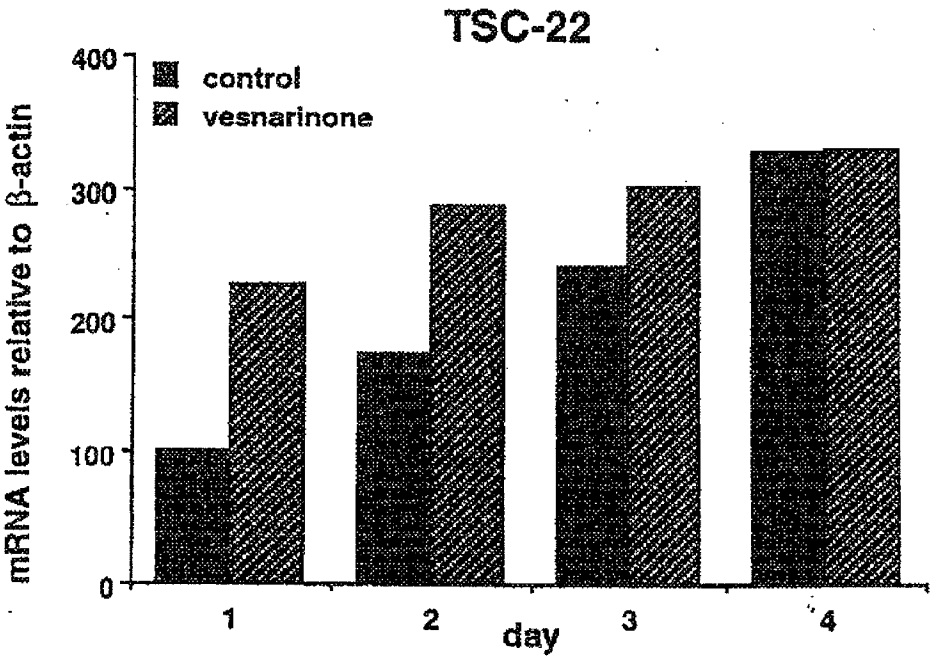
[FIG. 3]

A

drawing substituting photograph



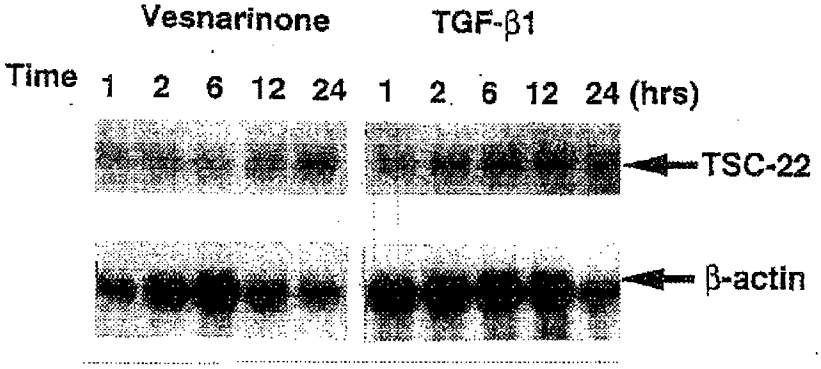
B



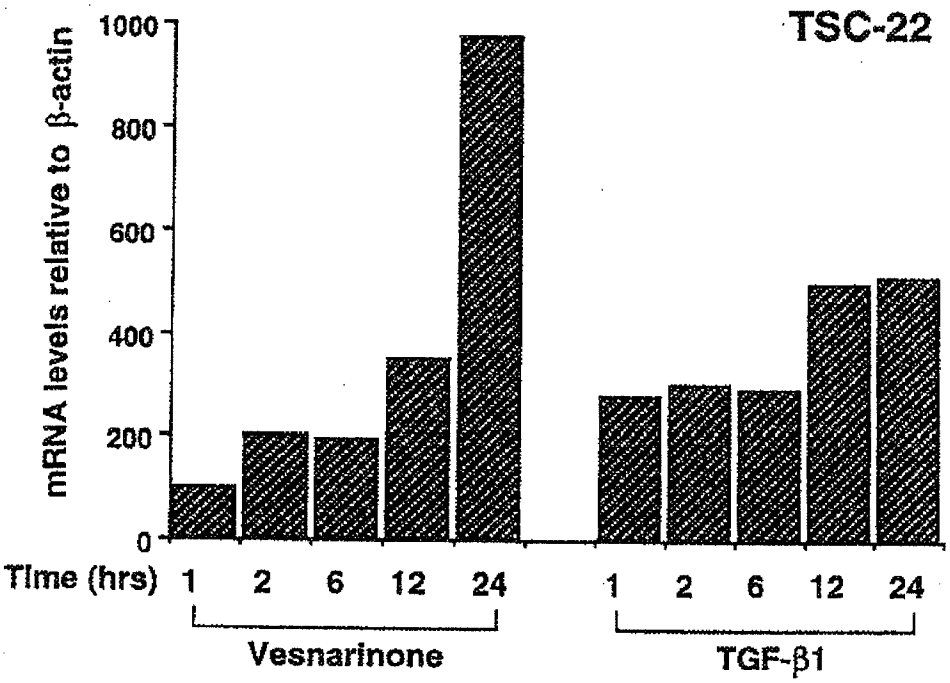
[FIG. 4]

A

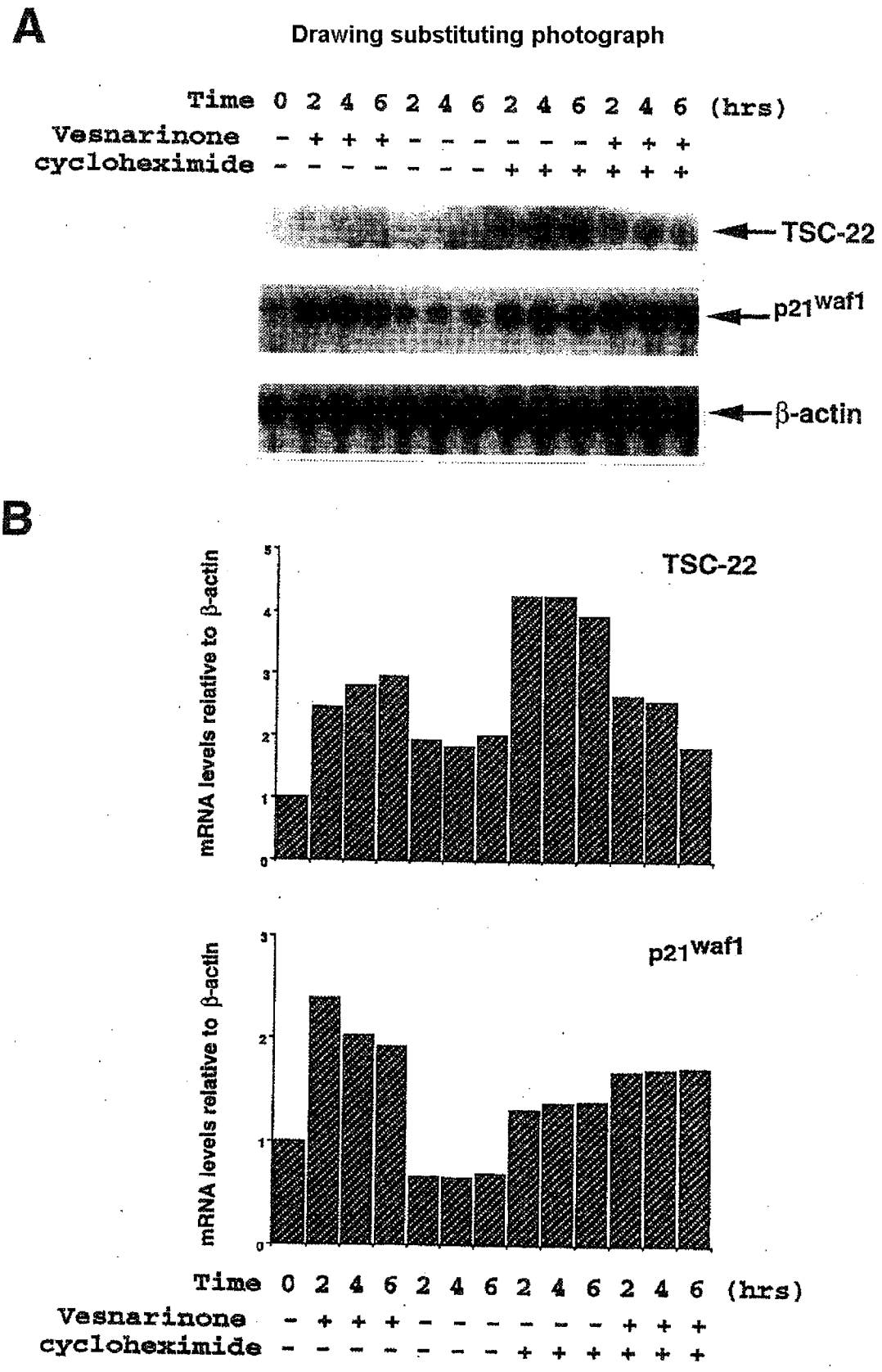
Drawing substituting photograph



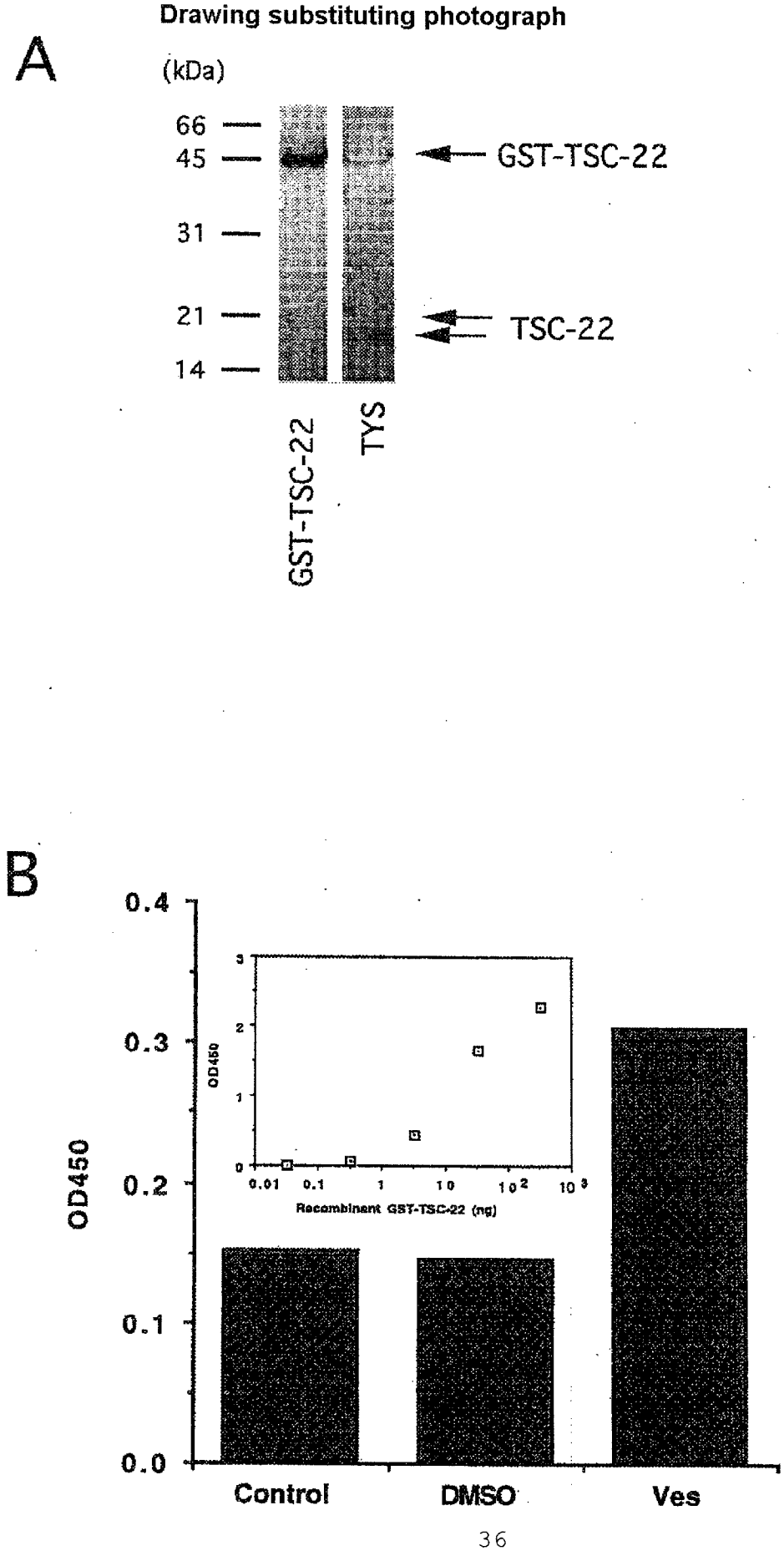
B



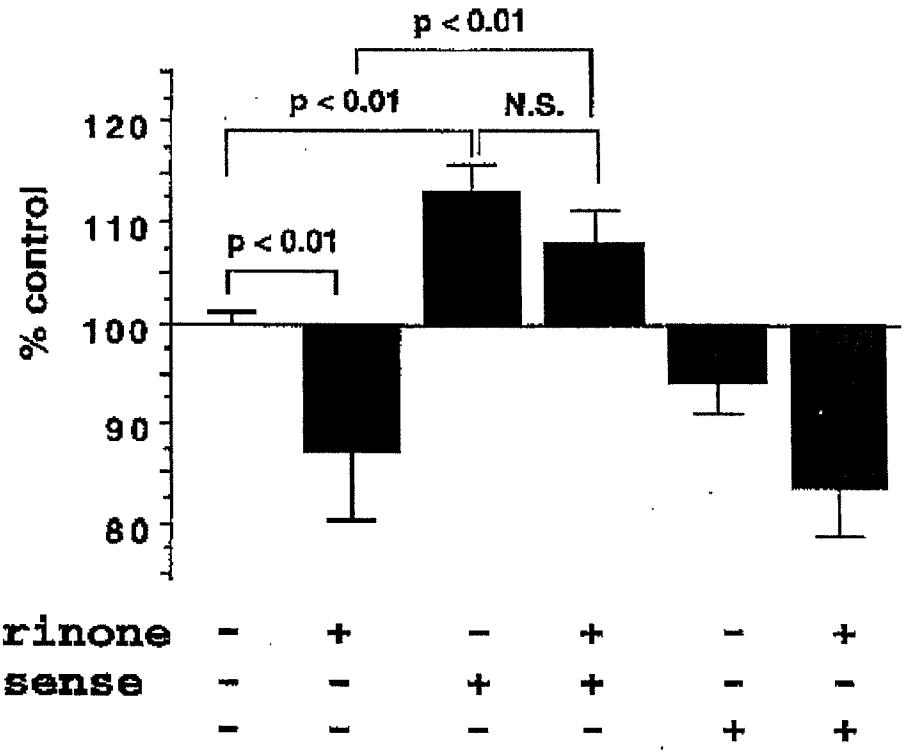
[FIG. 5]


A
Drawing substituting photograph
Time 0 2 4 6 2 4 6 2 4 6 2 4 6 (hrs)
Vesnarinone - + + + - - - - - - + + +
cycloheximide - - - - - - - + + + + + +
TSC-22
p21waf1
β-actin
B
TSC-22
mRNA levels relative to β-actin
p21waf1
mRNA levels relative to β-actin
Time 0 2 4 6 2 4 6 2 4 6 2 4 6 (hrs)
Vesnarinone - + + + - - - - - - + + +
cycloheximide - - - - - - - + + + + + +

[FIG. 6]


Drawing substituting photograph
A
(kDa)
66
45
31
21
14
GST-TSC-22
TSC-22
GST-TSC-22
TYS
B
OD450
0.4
0.3
0.2
0.1
0.0
OD450
Recombinant GST-TSC-22 (ng)
Control
DMSO
Ves

[FIG. 7]


p < 0.01
p < 0.01
N.S.
p < 0.01
120
110
100
90
80
% control
Vesnarinone - + - + - +
Anti-sense - - + + - -
Sense - - - - + +